**<Passaging>**

We can use **trypLE** similar to 2D cells, after aspirating the media. **1mL/well.**

The exposure time is **3-7min usually**. We can expose it to the organoids either in the 15mL tubes or in the plates. In the plates we need longer exposure time than in the **tubes in water bath**.

We don’t have to get single cells, because the organoid cells don’t want to be alone…they will easily die if they get alone.

The exposure time really depends on the Matrigel condition.

If the Matrigel is highly digested (=used) by the cells, maybe we need just 3 minutes.

We can see the residual Matrigel if we observe carefully. 🡪 Look for cloudiness or cell pellets at the bottom as opposed to large clumps/spheres in solution

After trypsinization, **add media (same 3x media to trypsin ratio) and spin down** same as 2D cells.

We can’t count the number of cells with cell counter.

Instead, we usually **passage them as 1:2 – 1:10**, depending on the next step or the next experiments or our holidays.

If we passage as **1:4,** usually the next time to **passage is after 3-4 days.**

After spinning down and aspiration of the supernatants, **resuspend** the organoid pellets **with Matrigel with tips primed with some media** (to avoid Matrigel sticking to the tips).

We don’t have to aspirate all of the supernatants. The final concentration of Matrigel could be >50%.

We have to aspirate the supernatant very carefully, because sometimes the pellets are not well attached to the bottom.

After resuspend with Matrigel, **make Matrigel domes as 15µL/each in NUNC 6-well plates**. (we can also use plates from other companies but NUNC plates are the best to get good shape of domes.)

🡪 Use enough matrigel to put 7-10 bubbles in however many 6-well plate wells are needed for the desired dilution; ex: 300uL for a 1:3 dilution (1 well to 3 wells) gives about 7, 15uL orbs per well

Wait for **10+ minutes to solidify** and **add the conditioned media in each well as 2.5mL/well**. The more is not always the better because the growth factor from the Matrigel will be diluted. Please don’t forget to add media, but even if we forget it we can still try to rescue them the next morning. (Nilay did!)

**<Thawing>**

Same as 2D cells.

Thaw quickly in 37C waterbath, add ~10mL media (wash buffer), spin down, resuspend with new matrigel, and plate them.

**<Freezing down>**

Almost same as 2D cells.

Aspirate media and add cryopreservation medium (FBS+DMSO or Cellbanker or something like that) and scrach the matrigel domes.

We don’t have to trypsinize.

Use Mr. Frosty same as 2D cells.

**<Conditioned media>**

We have made and use them as 50% conditioned media (= 1:1 mixture of collected media from WRN cells and DMEMF12 20%FBS).

To get rid of WRN cells (this is like fibroblasts), we need either spin down of the media or filtration.

I prefer filtration using 0.45µm filter, because this also reduces the risk of contamination.

Previously I filtered before storing in -80C, but will change to filter after thawing from -80C (=store them as unfiltered) just because other people are doing so.

Original protocol: Miyoshi et al. Nat Protoc. 2013 December ; 8(12): 2471–2482.

**Collect Protein from Organoids:**

1. Remove spent media, Wash the domes with PBS.
2. Add 1ml Corning Cell Recovery solution to the domes, pipette and disperse Matrigel. Collect the organoids into a 15 ml Falcon. Add 2 ml more Corning Cell Recovery solution.
3. Incubate on Ice for 40 minutes. Mix every 10 minutes by tapping and inverting.
4. Spin at 1000 rcf, 5 minutes. Discard supernatant.
5. Add 1ml PBS, resuspend organoids. Transfer to a 1.5ml Eppendorf.
6. Spin at 1000 rcf for 5 mins. Discard supernatant.
7. Add 100-200ul RIPA Buffer (With protease and phosphatase inhibitors). Mix by pipetting. Incubate in cold room on a rocker for 30mins.
8. Spin at 13000 rpm, 15 mins.
9. Collect supernatant i.e the protein lysate.